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Adenosine Mediation of Presynaptic Feedback Inhibition of Glutamate Release

Dario Brambilla,[1] David Chapman, and Robert Greene*
Department of Psychiatry
University of Texas Southwestern Medical Center
Dallas, Texas 75390

Summary

Conditions of increased metabolic demand relative to metabolite availability are associated with increased extracellular adenosine in CNS tissue. Synaptic activation of postsynaptic NMDA receptors on neurons of the cholinergic brainstem arousal center can increase sufficient extracellular adenosine to act on presynaptic A1 adenosine receptors (A1ADRs) of glutamate terminals, reducing release from the readily releasable pool. The time course of the adenosine response to an increase in glutamate release is slow (τ > 10 min), consistent with the role of adenosine as a fatigue factor that inhibits the activity of cholinergic arousal centers to reduce arousal.

Introduction

In the mammalian CNS, the localized extracellular adenosine (AD) concentration is increased in a variety of conditions with a greater ratio of metabolite demand to metabolite availability, including hypoxia, hypoglycemia, high potassium levels, epileptiform activity, and high-frequency stimulation (reviewed in [Dunwiddie and Masino, 2001; Greene and Haas, 1991]). Further, in neurons and in cardiac and smooth muscle cells expressing A1 adenosine receptors (A1ADRs), adenosine reduces excitability and thus may act as a negative feedback control to maintain a homeostasis between the local metabolic state and electrophysiological activity (Haas and Greene, 1984; McIlwain and Poll, 1986).

The cholinergic arousal centers, including the laterodorsal tegmental (LDT) and pedunculopontine tegmental nuclei of the brainstem and nuclei of the basal forebrain, are under tonic inhibitory control of adenosine acting through A1ADRs (Arrigoni et al., 2001; Rainnie et al., 1994). Accordingly, local metabolic changes that increase extracellular adenosine can reduce global cholinergic tone and facilitate the transition from waking to sleep (Portas et al., 1997), suggesting that adenosine may act as a fatigue factor in this respect. Microdialysis studies have provided evidence that in the hippocampus and striatum exploratory behavior and motor activity, respectively, are sufficient to increase extracellular adenosine (Huston et al., 1996). Prolonged waking is sufficient to increase adenosine in the basal forebrain cholinergic center, consistent with the role of adenosine as one of the brain's fatigue factors (Porkka-Heiskanen et al., 1997). This raises the question of what mechanism might be responsible for a sustained increase in extracellular adenosine under conditions of physiologically increased neuronal activity. Further, it may be asked if this kind of physiological increase in extracellular adenosine is consistent with the potential role of adenosine in the maintenance of a cellular homeostasis between the energy demands of neuronal activity and neuronal activity itself. This homeostasis might occur both at the cellular level, due to the convergence of diverse inputs to LDT neurons, and at a more global level, due to the divergence of these neurons' projections that supply the majority of the cholinergic tone of the brainstem and thalamus.

There is substantial evidence suggesting that adenosine modulates excitatory amino acid neurotransmission and vice versa, raising the possibility of a role for this interaction in the homeostatic coupling of energy metabolism to neuronal excitability. It is well documented that adenosine decreases presynaptic neurotransmitter release (Arrigoni et al., 2001; Fredholm and Dunwiddie, 1988; Poli et al., 1991; Prince and Stevens, 1992; Scanziani et al., 1992). It has been shown in both the hippocampus and the nucleus accumbens that the activation of the glutamatergic NMDA receptor (NMDAR) can cause the release of sufficient adenosine to exert a transient A1ADR-dependent inhibition of synaptic activity (Harvey and Lacey, 1997; Manzoni et al., 1994; Mitchell et al., 1993), suggesting that this adenosine-mediated inhibition may provide an inhibitory threshold against glutamate-mediated neurotransmission (Craig and White, 1993; Manzoni et al., 1994; White et al., 1993).

This study shows that in the LDT increased glutamate release within physiological parameters can increase extracellular adenosine in an NMDAR-dependent manner. The increase in adenosine is sufficient to feedback on to glutamate terminals and to cause a slowly developing presynaptic inhibition of the glutamate release.

Results

Spontaneous miniature synaptic events mediated by glutamatergic AMPA receptors (Arrigoni et al., 2001) were recorded from LDT neurons of Long Evans hooded rats, in vitro, in the presence of TTX (1 μM) as small inward, transient currents (occasional $GABA_A$ antagonist-sensitive outward mIPSCs were also observed). The mEPSCs were eliminated by either DNQX (20 μM) (Arrigoni et al., 2001) or NBQX (10 μM; n = 3). We assessed the effects of bath application of NMDA (10 μM; n = 45) on these spontaneous mEPSCs at a holding potential of −60 mV. NMDA decreased the mean mEPSC frequency (6.57 ± 0.86 Hz in control; 4.97 ± 0.58 Hz in NMDA; paired Student's t test, $p < 0.001$). The change in holding current response to NMDA application was less than 10 pA of inward current, with no significant change in whole-cell conductance. A significant de-

*Correspondence: robertw.greene@utsouthwestern.edu
[1]Current Address: Institute of Human Physiology II, University of Milan Medical School, Via Mangiagalli 32, I-20133 Milano, Italy.

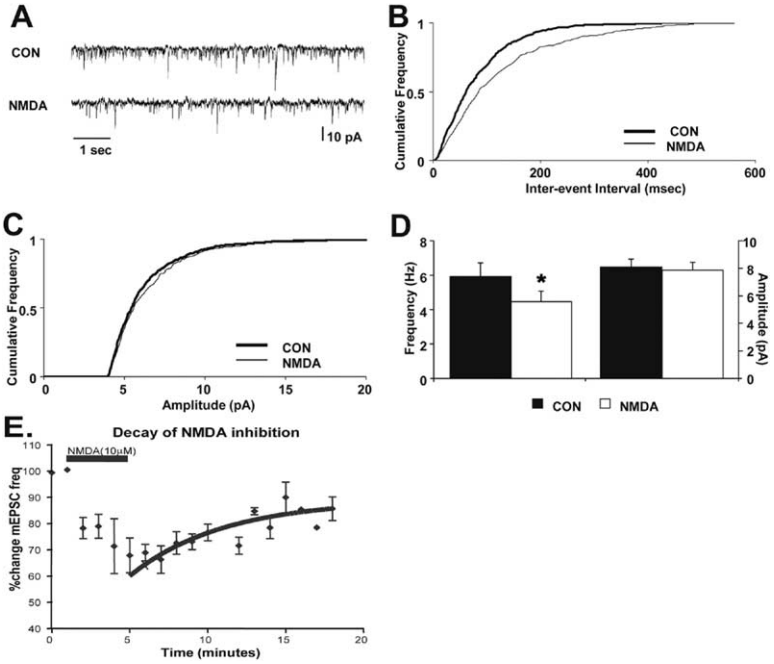

Figure 1. NMDA Can Reduce mEPSC Frequency in the Absence of Postsynaptic Electrophysiological Effects

(A) Continuous recordings of spontaneous mEPSCs in the presence of TTX under control conditions appear more frequent than those recorded in the presence of NMDA (10 μM). (B) A cumulative frequency histogram shows a righthand shift for interevent interval, as suggested by the continuous recordings. (C) The change of frequency was not accompanied by a change in amplitude distribution of the mEPSCs, nor was any change in input resistance observed in this cell. (D) Pooled data from all neurons without a change in mEPSC amplitude (n = 32) show a significant ($p < 0.001$) decrease in mean frequency. (E) The time course of NMDA-mediated reduction of mEPSC frequency and recovery elicited by a 4 min exposure (10 μM; n = 3) is shown as a percentage change from baseline (100%). The curve was derived from a single exponential best fit to the decay phase after the removal of the NMDA. $\tau = 6.0$ min; $R^2 = -1.01$. Error bars in (D) and (E) represent the mean ± SEM.

crease of the mean mEPSC amplitude (8.31 ± 0.48 pA in control; 7.98 ± 0.47 pA with NMDA; paired Student's t test; $p < 0.05$) was observed in a subset of the neurons examined (13 of 45). However, 32 of 45 cells did not show a change in mean mEPSC amplitude, nor were the cumulative amplitude distribution plots collected from these 32 neurons significantly altered from control by exposure to NMDA ($p > 0.1$, Kolmogorov-Smirnov test; events analyzed = 384 in control and 252 with NMDA). Within this population, mEPSC frequency was reduced from a mean of 5.87 ± 0.85 Hz, in control, to 4.49 ± 0.60 Hz in the presence of NMDA (paired Student's t test, $p < 0.001$; Figures 1A, 1C, and 1D). Finally, the reduction of the mean frequency correlates with a rightward shift in the distribution curve of interevent intervals ($p < 0.001$, Kolmogorov-Smirnov test; Figure 1B), further suggestive of a selective reduction in release probability (Redman, 1990).

The onset of the NMDA-dependent reduction of mEPSC frequency was relatively rapid, reaching 90% of the maximal effect within 4 min. At 4 min of exposure, mEPSC frequency was reduced to 67.9% ± 3.2% (n = 6) of the preexposure frequency. Continued exposure to NMDA (20 min) in three of six cells resulted in a further reduction of frequency to 63.3% ± 3.4% of baseline. For the remaining three cells, the NMDA was removed after 4 min, and the time course of recovery was examined (Figure 1E). The recovery data could be fitted with a curve described by a single exponential: $\tau = 6.0$ min; $R^2 = -1.01$.

Since pharmacologically identified glutamatergic synaptic activity in LDT neurons was shown to be presynaptically inhibited by adenosine (Arrigoni et al., 2001), we examined whether NMDAR activation was sufficient to exert a similar adenosine-mediated inhibition in the LDT. The A1ADR antagonist, 8-cyclopentyltheophylline (CPT; 1 μM) was bath-applied in the presence of TTX (1 μM; $V_h = -60$ mV) before the addition of NMDA (10 μM; n = 11). The CPT reduced or blocked the presynaptic inhibitory effect of NMDA (Figure 2). In seven of the 11 cells, NMDA was applied for 4 min, prior to application of the CPT to elicit a reduction of mEPSC frequency of 29.01% ± 3.29% (measured at 4 min of NMDA exposure). In these same neurons, after recovery from the NMDA exposure, the application of CPT alone (1 μM) had no effect. However, in the presence of CPT, the inhibitory effect of a new application of NMDA was antagonized (8.53% ± 6.50% reduction of mEPSC frequency; measured at 4 min of exposure to NMDA), consistent with a necessary role for A1ADR receptor activation in the NMDA-induced presynaptic inhibition.

Cumulative distribution curves of mEPSC interevent interval and amplitude during CPT application (10 min) and during application of CPT + NMDA (4 min) are indistinguishable (Figures 2B and 2C; thick line versus thin line, respectively). There are no significant changes in either mean mEPSC amplitude ($p > 0.1$, Kolmogorov-Smirnov test; events analyzed = 330 during CPT application and 315 with CPT + NMDA) or mean interevent intervals ($p > 0.1$, Kolmogorov-Smirnov test). The percentage change in mEPSC frequencies for each recorded neuron was calculated from the ratio of control to delayed control, obtained 4 min after the control, and the ratio of control to NMDA after 4 min of application of the NMDA for each neuron (>75% of maximal NMDA-induced inhibition was reached within 4 min of exposure; Figure 1E). These data were plotted as a distribution histogram showing the percentage of the total recorded neuronal population (for each condition) as a function of the percentage changes (Figure 2E). Similarly, distribution histograms were obtained for the per-

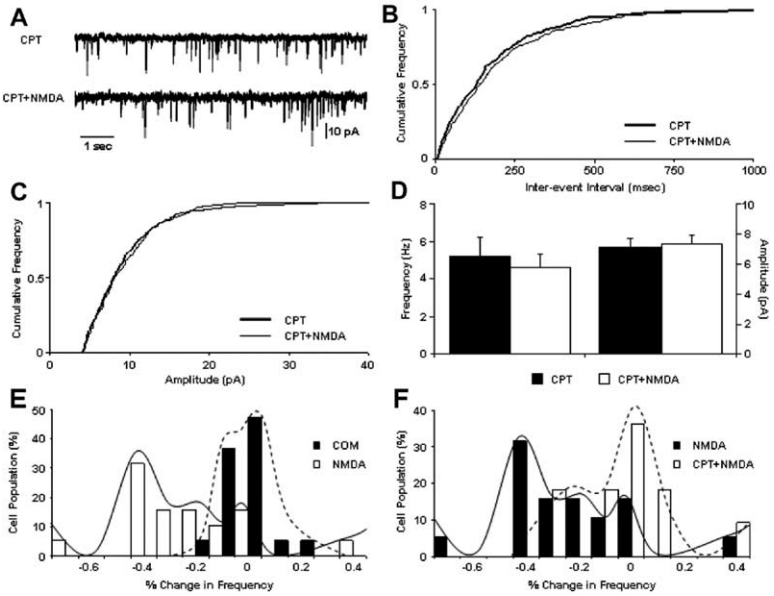

Figure 2. CPT Antagonizes the NMDA-Induced Decrease in mEPSC Frequency

(A) Continuous current recordings in the presence of CPT were not affected by the addition of NMDA (10 μM) to the perfusion media.

(B–D) Neither the cumulative frequencies for interevent intervals (B) nor the amplitudes (C) or pooled average frequency (D) was affected by NMDA in the presence of CPT (1 μM). Error bars in (D) represent the mean ± SEM.

(E) Further, the leftward shift in the population distribution of percentage change from control over time compared to the percentage change evoked by NMDA (over the same time) is similar to the leftward shift of NMDA + CPT to NMDA (F).

centage change from control to NMDA application and that from NMDA to CPT + NMDA applications (Figure 2F). NMDA application shifted the distribution of percentage change in mEPSC frequencies to the left compared to control; however, in the presence of CPT, the NMDA-elicited shift was antagonized, as suggested by a comparison of Figures 2E and 2F showing similar distributions for control and CPT + NMDA. This further indicates that the NMDA-induced changes are sensitive to the A1ADR antagonist CPT.

Evoked EPSCs mediated by AMPA receptor activation in the LDT were inhibited by exogenous adenosine as well as by an increase in endogenous adenosine resulting from inhibition of adenosine kinase (Arrigoni et al., 2001). In the presence of picrotoxin (50 μM), evoked EPSCs were reversibly reduced in amplitude by 48% ± 8% (n = 4) following the addition of NMDA (10 μM) to the perfusion media (Figure 3A). With continuous NMDA exposure the inhibition of the evoked EPSC was completely reversed by CPT in the same cells.

Although the inhibition was completely reversed by CPT, there was an apparent lack of CPT-mediated enhancement of the EPSC (due to removal of endogenous adenosine tone) that was previously observed with exposure to CPT alone (Arrigoni et al., 2001). We found a trend toward increase of the evoked response compared to the control EPSC amplitude but the effect did not reach significance (Figure 3B). Neither CPT (n = 11) nor adenosine deaminase (ADA) (n = 2) alone were able to increase the mEPSC frequency over that in controls, although the variability of the mean did increase. When mEPSC frequency was increased by increased osmotic pressure, a further increase was induced in the presence of CPT (see Figure 6). Since the mEPSC frequencies were determined in the presence of TTX with an expected reduction of glutamate release relative to its release in the absence of TTX, this is not inconsistent with the findings of Arrigoni et al. (2001) in the absence of TTX.

The enzyme adenosine deaminase catalyzes the deamination of adenosine to inosine (Zimmerman et al., 1995). In the presence of TTX (1 μM; V_h = –60 mV), the addition of ADA (0.8 IU/ml), blocked the NMDA (10 μM; n = 5) effect, much as the CPT application had (Figure 4). There were no significant changes observed in the cumulative distribution of either mEPSC amplitude ($p > 0.1$, Kolmogorov-Smirnov test; events analyzed = 253 during ADA application and 210 with ADA + NMDA) or interevent intervals ($p > 0.1$, Kolmogorov-Smirnov test), or in the mean amplitude (7.25 ± 0.83 pA in control; 7.23 ± 0.79 pA with ADA + NMDA; paired Student's t test; $p = 0.907$) or frequency (2.87 ± 0.44 Hz in control; 2.60 ± 0.40 Hz with ADA + NMDA; paired Student's t

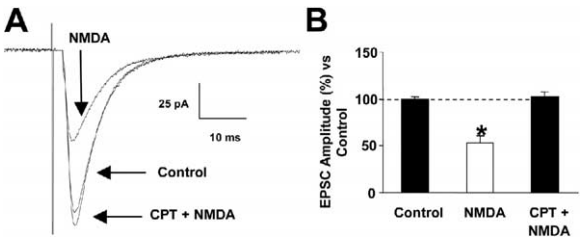

Figure 3. The NMDA-Induced Decrease in Evoked EPSC Amplitude Is Abolished by CPT

(A) Application of NMDA (10 μM) reduces the evoked EPSC amplitude, which can be blocked by application of CPT (1 μM), as indicated by sample traces showing the peak amplitude of EPSCs from neurons in rat LDT. Traces represent the averages of 15 EPSCs evoked at 0.1 Hz. Currents were recorded at a membrane potential of –60 mV in the presence of 50 μM picrotoxin.

(B) Pooled data from all neurons show a significant change in evoked EPSC amplitude in the presence of NMDA. Black bars represent control (left) and results following application of CPT and NMDA (right). The open bar represents conditions following NMDA application. The y axis indicates the evoked EPSC peak amplitude expressed as a percentage of control. Each bar represents the mean ± SEM of ≥ 4 cells.

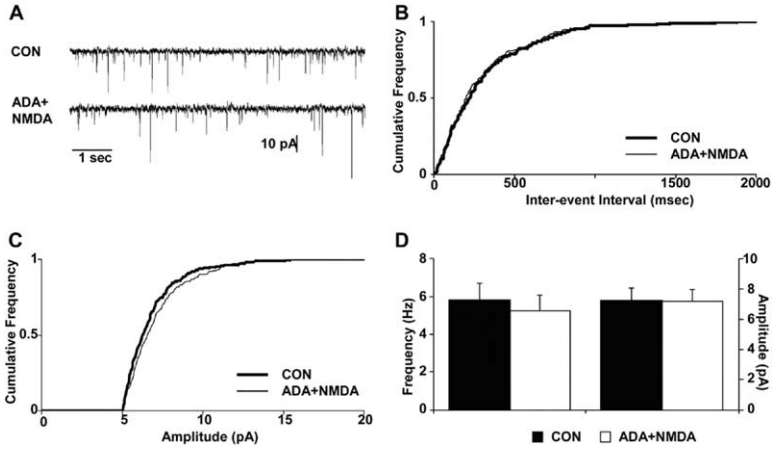

Figure 4. A Catabolic Enzyme of Adenosine, ADA, Reduces the NMDA-Induced Decrease in mEPSC Frequency

(A) Continuous current recordings in the presence of ADA (0.8 IU/ml) were not affected by the addition of NMDA (10 μM) to the perfusion media.

(B–D) Neither the cumulative frequency curve (B) nor the amplitude distributions (C) or pooled average frequency (D) was affected by NMDA in the presence of ADA. Error bars in (D) represent the mean ± SEM.

test, p = 0.178). On two of the five cells, NMDA was tested before the application of ADA + NMDA, with a resultant significant reduction of mEPSC frequency.

The adenosine-dependent presynaptic inhibition was evoked by the selective NMDAR agonist NMDA. The necessary involvement of the NMDAR was confirmed by blockade with aminophoshonovalerate (APV) or MK801 (APV, 20 μM, n = 2; MK801, 10 μM, n = 3). All cells tested with MK801 had been tested beforehand with NMDA (10 μM) alone and responded to the NMDA with reduced mEPSC frequency as described above.

Since MK801 is an open-channel blocker that does not prevent binding of glutamate to the NMDAR (Hessler et al., 1993; Rosenmund et al., 1993), it can be used as a tool to examine the effect of decreased NMDAR conductance, even during glutamate binding. Its application prevented NMDA-induced mEPSC frequency reduction (Figures 5A–5C), suggesting that, in addition to glutamate binding, ionic flux across the NMDAR channel is necessary.

Since the NMDAR channel is permeable to calcium, the role of NMDA-induced intracellular calcium increase was examined by the addition of EGTA to the patch-pipette recording solution (3 mM; n = 9) or by the addition of BAPTA (10 mM; n = 4). Neither had any effect on NMDA-induced presynaptic inhibition, suggesting that an increase in intracellular calcium in the same neuron that shows presynaptic inhibitory effects is not necessary for the NMDA-induced decrease in mEPSC frequency. Nevertheless, this does not rule out the possibility that the increase in adenosine might be dependent on NMDAR-mediated calcium-dependent effects in other surrounding cells (including glia).

Calcium flux across the cellular membrane can be reduced by removing calcium from the ACSF (Mg^{2+} = 3.5 mM). Under these conditions, the mEPSC frequency was not significantly altered, but NMDA no longer had an effect on either mEPSC frequency or amplitude (n = 3; Figures 5D–5F), suggesting that the NMDA-mediated increase in adenosine is dependent on calcium flux across cellular membranes, even if it is not necessarily dependent on calcium increases in the neuron showing presynaptic inhibition.

The presynaptic actions of adenosine in the LDT are not mediated by an activation of GIRK (G protein-activated potassium) conductances (Arrigoni et al., 2001) or by an inhibition of calcium channels, as these are not activated in TTX. Adenosine can reduce the hyperpolarization-activated current Ih in LDT neurons (Rainnie et al., 1994), most likely by a leftward shift of V1/2, as observed in thalamic neurons (Pape, 1992). Presynaptic activity may be modulated by Ih (Yu et al., 2004) and thus, a potential mechanism of adenosine-mediated presynaptic inhibition could involve this current. However, the addition of an antagonist of Ih, ZD 7288, (50 μM; n = 3) had no effect on either the decrease in NMDA-induced mEPSC frequency or on amplitude. Further, ZD 7288 had no effect on baseline mEPSC parameters, suggesting that Ih is not involved in the NMDA-induced, adenosine-mediated presynaptic inhibition.

The spontaneous quantal release of neurotransmitter, recorded as mEPSCs in the presence of TTX, is increased by raised osmotic pressure of the extracellular medium. The increase in release has been used to determine the size of a readily releasable pool (RRP) of neurotransmitter in glutamatergic systems that corresponds to the RRP released by action potentials (Pyle et al., 2000; Rosenmund and Stevens, 1996; Sara et al., 2002; Stevens and Sullivan, 1998; Stevens and Tsujimoto, 1995). Typically, upon application of the medium high in osmotic strength (100–500 mOsm), quantal frequency first increases to a maximum and then decreases exponentially to a new steady state in less than 5 s. The decrease is thought to reflect the depletion of the RRP.

We examined the effect of increasing osmotic pressure by adding 50 mM of sucrose in the presence of TTX and found that, within 10 min of exposure, the increase in frequency of mEPSCs reached a maximal plateau (Figures 6A and 6B; n = 4), reflecting the maximal effect on the RRP. After an additional 10 to 15 min, the mEPSC frequency returned to baseline with a time constant of 11.0 ± 0.3 min (Figure 6B). Neither the amplitude distributions of the mEPSCs nor the input resistance was altered over the 30 min recording period.

The slower kinetics of the increase to maximal effect, compared with those observed in the aforementioned

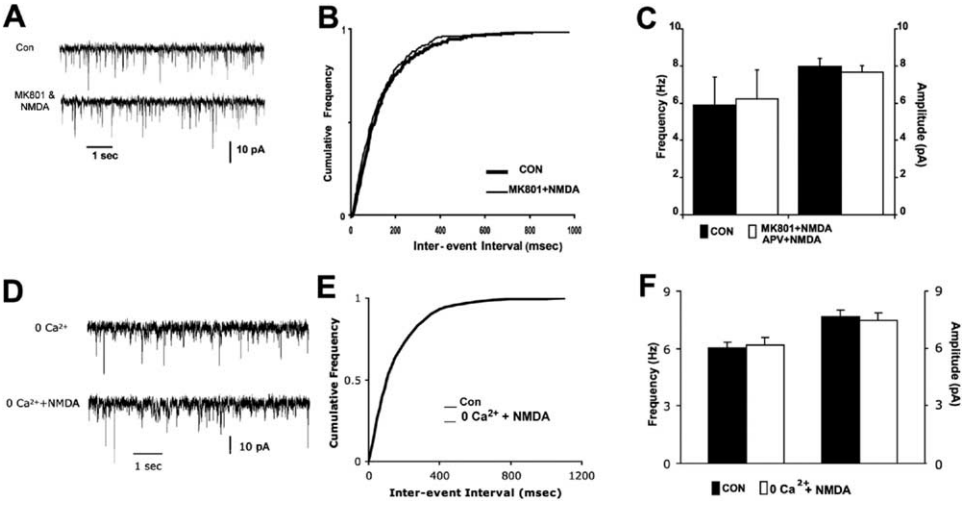

Figure 5. APV and MK801 Antagonize the NMDA-Induced Decrease in mEPSC Frequency, as Does the Removal of Calcium from the Extracellular Medium

(A) Continuous current recordings in the presence of MK801 were not affected by the addition of NMDA (10 μM) to the perfusion media.
(B and C) Neither the cumulative frequency curve (B) nor the pooled average frequencies or amplitudes (C) were affected by NMDA in the presence of MK801 (1 μM) or APV (20 μM). Error bars in (C) represent the mean ± SEM.
(D) Continuous current recordings in the absence of calcium in the perfusion medium were unaffected by the addition of NMDA (10 μM).
(E and F) The cumulative frequency curve (E) and pooled average frequencies and amplitudes (F), were unaffected by NMDA in the absence of calcium. Error bars in (F) represent the mean ± SEM.

culture preparations, may be due to diffusion barriers and osmotic buffering not present in the culture preparations. The slow kinetics of the decay that follows the steady-state maximal plateau are inconsistent with mediation by depletion of the readily releasable pool, as the depletion occurs within seconds (Rosenmund and Stevens, 1996). A slow, negative feedback mechanism may exist to limit the synaptic release of glutamate, in response to the sustained increase in glutamate release.

Since synaptic glutamate release can activate NMDARs, and NMDAR activation can induce an increase in electrophysiologically active extracellular adenosine, we examined the effects of the NMDAR antagonist APV (20 μM; n = 4) and the A1ADR antagonist CPT (1 μM; n = 4) on the slow decay of the mEPSC frequency. The antagonists were applied in separate experiments, more than 10 min before the osmotic strength was increased. The effects of either of the antagonists became apparent with the onset of the hyperosmotically evoked increase of mEPSC frequency, suggesting a rapid onset of NMDA-evoked inhibition of adenosine. Either antagonist was sufficient to block the slow decay (further evidence that the decay was not due to a nonspecific rundown; Figure 6B). After 20 min of exposure to the high osmotic pressure, the ratio of

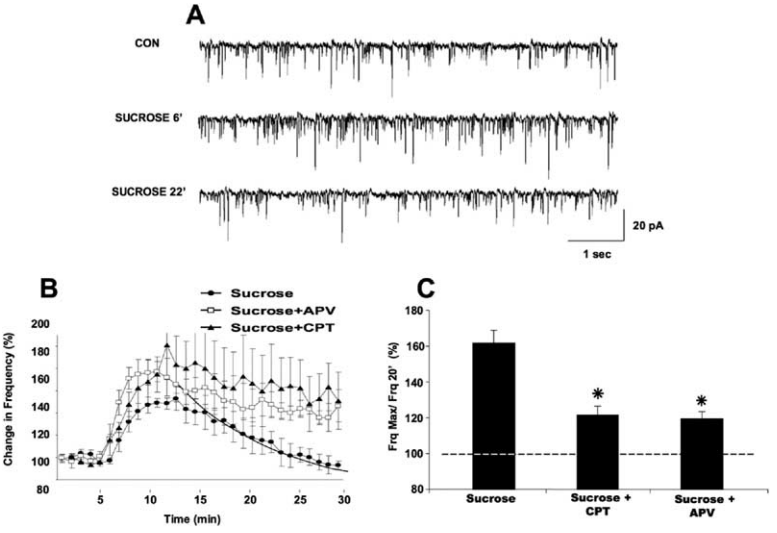

Figure 6. An Increase in Synaptic Release of Glutamate Is Limited by Activation of A1ADRs

(A) Three segments of a continuous current recording of a LTD neuron in TTX show mEPSCs during control conditions (top), after application of hypertonic ACSF for 6 min (middle), and after ACSF application for >20 min (bottom). Spontaneous mEPSC frequency was initially increased and then decreased.
(B) Pooled data show the percentage change in frequency from control as a function of duration of hypertonic ACSF application (n = 4). Error bars represent the mean ± SEM.
(C) The ratio of the maximum mEPSC frequency to the frequency recorded after 20 min of exposure to hypertonic media shows antagonism by either APV or CPT of the decay of the hypertonically induced frequency increase. Error bars represent the mean ± SEM.

the mEPSC frequency to the maximum frequency elicited was calculated for each cell under control and in the presence of either CPT or APV. The pooled results show the effective blockade of the decay by either of the agents (Figure 6C). These data suggest that the slow decay of mEPSC frequency that occurs in response to an increased glutamate release is mediated by an NMDAR-dependent increase in adenosine activity at presynaptic A1 receptors, which acts to inhibit the release of glutamate, consistent with homeostatic control of synaptic glutamate release.

Discussion

Adenosine exerts a presynaptic inhibitory tone in the forebrain of the mammalian CNS mediated by A1ADRs (Dunwiddie and Masino, 2001). NMDA receptor (NMDAR) activation can increase sufficient extracellular levels of adenosine (Craig and White, 1993; White, et al., 1993) to significantly increase this inhibitory tone (Manzoni et al., 1994; Mitchell et al., 1993). The present study shows that in the brainstem cholinergic arousal center, the LDT, a sustained increase of synaptically released glutamate can cause increased NMDAR activation that, in turn, causes an increase in extracellular adenosine. Notably, the magnitude of the increased, spontaneous synaptic activity elicited in this study was well within physiological parameters, and, under these conditions, an increase of extracellular adenosine sufficient to cause a feedback A1ADR-mediated presynaptic inhibition of glutamate synaptic activity was induced. Thus, adenosine can mediate a negative feedback regulation of synaptic glutamate release.

Synaptic Release of Glutamate, NMDA Receptor Activation, and Adenosine Release

In the LDT, an increase in A1ADR inhibition may occur with modest changes in synaptic release of glutamate even in the presence of TTX, suggesting that, in vivo, a dynamic inhibitory A1ADR-dependent tone is normally present. The A1ADR inhibition in response to a sustained increase in synaptically released glutamate (in the present study, the increase was induced by sustained exposure to a hyperosmotic medium) occurs at a slow rate with a time constant of greater than 10 min (Figure 6B), consistent with a physiological role in the behavioral state transition from waking to sleep.

The increase in NMDAR conductance caused by the addition of NMDA to the bath medium resulted from an increase in extracellular adenosine, as suggested by the blockade of the inhibition by either ADA or CPT. The NMDAR activation caused an increase of inward current and an influx of Ca^{2+}. The observed increase in current was too small to cause either the activation of voltage-gated calcium channels as shown under current-clamp conditions (the addition of 10 μM of NMDA to the bathing medium evoked a change of membrane potential of < 4 mV in all cases) or to affect energy metabolism significantly. Thus, the most likely mechanism mediating the increase in adenosine is an influx of ions through NMDAR channels (probably Ca^{2+}) that alters adenosine metabolism.

Evidence for an inhibitory adenosine tone, revealed by the excitatory effects of either CPT or ADA, was reported in the LDT (Arrigoni et al., 2001; Rainnie et al., 1994). In the present experiments, the lack of significant enhancement with evoked EPSCs in the absence of TTX (Figure 3) was probably due to the additional presence of NMDA along with the CPT. The CPT antagonism of the NMDA effect may have been incomplete. Additionally, it is likely that some escape from voltage-clamp control at the synapse occurred (Hausser and Roth, 1997), resulting in an enhanced voltage-sensitive conductance of NMDA-dependent currents, including Ca^{2+} and, accordingly, Ca^{2+}-dependent potassium current. The latter would shunt the evoked EPSC, consistent with the lack of CPT-induced enhancement in the presence of exogenous NMDA. Nevertheless, in the present experiments we did observe that in the presence of TTX, when synaptically released glutamate was increased by exposure to a hyperosmotic medium, either CPT or APV, alone, did increase mEPSC frequency, suggestive of an NMDAR-induced inhibitory adenosine tone.

A1ADR Presynaptic Inhibition

Adenosine, acting through A1ADR receptors, causes a presynaptic inhibition of glutamate release in LDT neurons (Arrigoni et al., 2001). The location of the A1ADR mediating this effect is probably the presynaptic terminal. Studies employing immunohistochemical localization (Rivkees et al., 1995), in situ mRNA expression analysis (Reppert et al., 1991), or autoradiographic techniques (Goodman et al., 1983) all indicate the expression of A1ADR primarily in neurons and the presence of A1ADRs on neuronal processes and excitatory neuronal axonal processes in particular (Goodman et al., 1983). Nevertheless, A1ADRs are expressed in cultured astrocyte preparations (Biber et al., 1997), and a role for A1ADR-dependent presynaptic modulation by glia cannot be ruled out. If this modulation occurred, it is unlikely that it was an indirect effect of glial-dependent neuronal energy depletion, as glucose was included in the medium. Furthermore, the onset of the induced decrease in mEPSC frequency was relatively rapid (Figure 1E), most consistent with a direct effect on A1ADRs and not involving the glial-dependent neuronal energy depletion.

In the presence of TTX, a hyperosmotic solution induced an increase in neurotransmitter release, indicated by an increased frequency of mEPSCs. These mEPSCs were all mediated by fast inward currents and were indistinguishable from the glutamate-mediated spontaneous mEPSCs recorded in control conditions; however, additional mEPSCs mediated by different neurotransmitters may have contributed. In any case, these mEPSCs originated from a RRP that appears to be the same as that released by action potential generation (Pyle et al., 2000; Rosenmund and Stevens, 1996). The size of the RRP correlates with the amplitude of action potential-dependent EPSCs (Murthy et al., 1997; Wang and Zucker, 1998) and can be increased by diacyl-glycerol and/or β phorbol ester activation (Rhee et al., 2002) and decreased by PKC antagonism (Stevens and Sullivan, 1998). Our findings suggest that this same RRP may be reduced by NMDAR-dependent increases in extracellu-

lar adenosine. However, we cannot rule out an effect on the probability of vesicle release. Adenosine was shown to reduce the RRP at the frog neuromuscular junction, based on its inhibition of high-frequency action potential-dependent release of acetylcholine (Searl and Silinsky, 2003), although whether the reduction was due to adenosine-mediated changes in activity-dependent presynaptic calcium concentration was unclear. The increase in mEPSC frequency induced by exposure to a hyperosmotic medium is not dependent on intracellular calcium (Rosenmund and Stevens, 1996), consistent with a calcium-independent A1ADR effect. This would not rule out additional calcium-dependent effects, as reported at the mouse neuromuscular junction (Silinsky, 2004). Nonetheless, with either or both presynaptic adenosine mechanisms, the action that we have observed provides a mechanism for slow, local inhibitory feedback limiting the synaptic release of glutamate.

The Time Course of Adenosine-Mediated Feedback Inhibition

The onset of NMDAR activation-induced adenosine inhibition observed in CA1 pyramidal cells of the hippocampus was relatively fast, with a time course ranging from 10 ms to 2000 ms (Manzoni et al., 1994; Mitchell et al., 1993). The NMDAR activation in these studies resulted from tetanic trains of electrical stimuli applied to the Schaffer collateral, afferent pathways, and, thus, the adenosine response was to a transient activation. In the LDT, we also observed a rapid onset of presynaptic inhibition in response to bath application of NMDA (Figure 1E) as well as to a hypertonic stimulus (Figure 6B). With respect to the latter, the onset of the inhibitory adenosine effect was reflected by the initial difference between the two curves, one generated by the hypertonic medium alone and the other, by hypertonic medium plus CPT. While the rapid onset provides some idea of the timing of this response to a transient increase in neurotransmission, it does not indicate the time course of an equilibrated adenosine tone resulting from a sustained change in neurotransmission, as may occur with a change in arousal levels or in transition across sleep/wake states (Steriade et al., 1990). The time course of the response to a sustained change is influenced by the kinetics of both onset and offset of NMDAR-induced adenosine inhibition.

The hypertonic stimulus in the present study was applied continuously, causing an initial small but persistent drive for an increase in synaptic activity well within physiological parameters. The ensuing presynaptic inhibition was thus in response to a relatively small but sustained increase in activity. It resulted in a return to the prestimulus level of release frequency. The decay rate had a time constant of >10 min, probably reflective of at least two processes: (1) with increased NMDAR activation, the NMDAR-dependent increase in adenosine and (2) with decreased NMDAR activation, the decrease in extracellular adenosine. In response to increased synaptic activity, activation increased, increasing presynaptic inhibition by adenosine as confirmed by the sensitivity to both APV and CPT, and in response to the adenosine-mediated inhibition, NMDARs were less activated, reducing the NMDAR-dependent extracellular adenosine.

Functional Implications

In fact, extracellular adenosine levels have been observed to increase slowly in the basal forebrain cholinergic arousal center (Porkka-Heiskanen et al., 1997) with prolonged waking, a state correlating with increased glutamatergic activity. The observed increase with prolonged waking was slower than the changes in adenosine inhibition induced by tetanic stimulation in vitro (Manzoni et al., 1994; Mitchell et al., 1993) and probably reflected the slower approach to a new equilibrium between the increased synaptic drive during prolonged waking and the adenosine-mediated inhibitory feedback, which we observed in response to a sustained, hypertonically driven synaptic activity.

An adenosine-mediated feedback, limiting glutamate release in other regions of the CNS, may also occur. Local increases of adenosine in association with increased neuronal activity in hippocampal, cortical striate, and thalamocortical regions have been reported (Huston et al., 1996; Porkka-Heiskanen et al., 2000). Adenosine, acting through A1ADRs, may exert an inhibitory tone in each of these regions (Dunwiddie and Masino, 2001), and the increased neuronal activity is associated with increased glutamate release in most forebrain regions, although a specific role for NMDA receptors has not been demonstrated in these cases.

The increased adenosine inhibitory tone probably affects the functions under the control of the CNS area where it occurs. However, a change in behavioral state would not be expected to occur except for CNS regions that exert a more global influence, as behavioral state is a more global phenomenon involving many functions. If the increased inhibitory adenosine feedback occurs in the brainstem cholinergic arousal center, then a decrease in the cholinergic tone of the target areas, including the thalamus and brainstem, would be likely, facilitating the transition from waking to sleep, as observed when exogenous adenosine was locally microdialyzed into the LDT region (Portas et al., 1997). Thus, a localized negative feedback inhibition of glutamate synaptic activity that is mediated by adenosine is consistent with a NMDAR-dependent mechanism mediating the waking or arousal state-related increase in adenosine.

Experimental Procedures

In vitro slice preparation of the laterodorsal tegmental nucleus in 20- to 30-day-old Long Evans hooded rats was as previously described (Arrigoni et al., 2001; Luebke et al., 1993). Animals were anesthetized prior to the start of the procedure. Slices (300–400 μm) were cut by vibratome, placed in gassed (95% O_2/5% CO_2) artificial CSF (ACSF) at room temperature, and then placed in a submerged, oxygenated recording chamber and maintained at 32°C for the duration of the experiment. The ACSF contains: 124 mM NaCl, 2 mM KCl, 3 mM KH_2PO_4, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH = 7.35, after gassing with 95% O_2/5% CO_2) with 315–320 mOsm.

Recording

Standard whole-cell recording techniques were used with borosilicate glass micropipettes (outer diameter, 1.5 mm; inside diameter,

0.86 mm) pulled to a resistance of 4–10 MΩ when filled. The recording medium contained 120 mM KGluconate, 10 mM KCl, 3 mM $MgCl_2$, 10 mM HEPES, 2 mM MgATP, and 0.2 mM NaGTP (pH = 7.2, adjusted with KOH) with 280 mOsm. Miniature excitatory postsynaptic currents (mEPSCs) were observed in the presence of TTX, using continuous 60 s epochs of mEPSCs recorded with an Axopatch 1D amplifier (Axon Instruments, CA) with a 2 KHz low-pass filter. Data were digitized at 2–5 KHz with a Digidata 1200B data acquisition system controlled by Clampex 8 software (Axon Instruments, CA) and analyzed offline semiautomatically with Mini Analysis 4.0 software (Synaptosoft, NJ), after processing with a band pass of ~2 Hz to 1 KHz using a single-pole digital filter. Event selection was visually checked for all data, and then the events were ranked by amplitude and interevent interval for use in cumulative probability distribution plots. Population means are expressed as the mean ± SEM, and statistical analysis employed the nonparametric Kolmogorov-Smirnov (K-S test) or the paired Student's t test to ascertain significance.

Evoked EPSCs were elicited using local, minimal stimulation. Briefly, a biopolar stimulating electrode was placed in proximity to the recording electrode (< 200 μm). The stimulating electrode was used to deliver current pulses (150–300 μA, 100 μs duration) sufficient to produce a small EPSC (~50 pA), which could be reliably evoked at low frequency. To isolate EPSCs, picrotoxin (50 μm) was added to the ACSF solution to block $GABA_A$ receptors. Data were acquired with a MultiClamp Commander 700A amplifier and the Clampex 8 software package interfaced to a Digidata 1322A acquisition board and analyzed offline with AxoGraph 4.6 (Axon Instruments). Data were expressed as the mean of the control peak amplitude (± SEM), and the paired Student's t test was used to determine statistical significance. Signals were filtered at 4 kHz and sampled at 10 kHz. All reagents were obtained from Sigma/RBI.

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